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(54) Title: PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS (57) Abstract The present invention provides the use of natural, synthetic or modified peptide factors derived from murine epidermal growth factor in the treatment of angiogenic diseases by targeting laminin receptors. The invention provides agonists and antagonists which may be modified to prevent proteolytic degradation. Use of the invention to treat retinopathy of prematurity and promote wound healing is envisaged.		

**PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS
LAMININ RECEPTOR TARGETS**

1
2
3 This invention relates to the use of (synthetic and
4 modified) laminin receptor-targetted ligands for the
5 treatment of angiogenic diseases such as proliferative
6 retinopathies and metastatic cancer as well as for the
7 treatment of Candida spp. infections, or parastic
8 infestations such as leishmania and trichomonas
9 vaginalis.

10
11 Laminin antagonists (which are anti-angiogenic) can be
12 used to inhibit secondary tumour spread (by inhibiting
13 tumour cell attachment) and to prevent growth of
14 metastatic secondaries (by inhibiting
15 neovascularisation). These antagonists could also be
16 used to treat other angiogenic disorders (such as
17 diabetic retinopathy).

18
19 Laminin agonists (which promote angiogenesis) could be
20 used to treat retinopathy of prematurity, and could
21 also be used to promote wound healing (for example in
22 corneal epithelium).

23
24 Both the antagonists and the agonists would be expected
25 to inhibit parasite binding to tissue surfaces and
26 would thus prevent infection or infestation.

27
28 Angiogenic diseases are those disorders which are
29 directly caused by, or complicated by the inappropriate
30 growth of new blood vessels. The major angiogenic
31 diseases include the common metastatic solid tissue

1 cancers (breast, gastrointestinal, lung, prostatic,
2 etc), diabetic retinopathy, neovascular glaucoma,
3 rheumatoid arthritis and psoriasis. Angiogenesis is
4 the rate-limiting step in the growth of secondary
5 tumours; inhibition of their neovascularisation is
6 known to stop their growth.

7
8 In this field it is already known that the native
9 ligand of the 67kDa laminin receptor (67LR) is
10 encompassed by the linear sequence of amino acids 925-
11 933 of the laminin β -1 (previously known as laminin B1
12 or b1) chain (numbering refers to the mature murine
13 laminin β -1). Synthetic laminin β -1₉₂₅₋₉₃₃ (single letter
14 amino acid code: CDPGYIGSR-NH₂) has been shown to
15 inhibit tumour establishment in mice, by inhibiting
16 attachment of tumour cells to basement membranes. It
17 has also been demonstrated that laminin β -1₉₂₅₋₉₃₃
18 inhibits angiogenesis in the chick.

19
20 However, synthetic laminin-derived peptide (laminin
21 β -1₉₂₅₋₉₃₃) stimulates angiogenic events in mammalian
22 cells (in which it acts as a pure 67LR agonist), making
23 it useless as the basis of a human therapy.

24
25 It is one object of the present invention to provide a
26 medicament to treat angiogenic diseases.

27
28 The present invention provides a peptide factor derived
29 from murine epidermal growth factor (EGF) peptide for
30 use in the preparation of a medicament for the
31 treatment of angiogenic diseases.

32
33 The mechanism by which EGF derived peptides inhibit new
34 blood vessel formation is through their antagonism of
35 the high affinity 67 kDa laminin receptor (67LR) found
36 on endothelial cells.

1 The peptides have the additional effect of inhibiting
2 tumour cell attachment to basement membranes, and may
3 be used to prevent solid cancer spread in cases where
4 cancer cells have been identified circulating in the
5 blood.

6
7 Modified peptides may be protected from proteolytic
8 degradation by substitution of key residues with
9 unnatural amino acid analogues at susceptible bonds,
10 such as tyrosine analogues (at position 5) and arginine
11 analogues (at position 9). The peptides may be capped
12 at N- and C-termini (with acetyl and amide groups
13 respectively) and at the thiol groups of the cysteines
14 (with acetamido methyl groups).

15
16 Typically the peptide is an antagonist of the 67kDa
17 Laminin Receptor (67LR).

18
19 The peptide factor is based on amino acid residues 33
20 to 42 of murine epidermal growth factor (mEGF).

21
22 The amino acid sequence of mEGF- (33-42) is CVIGYSGDRC.

23
24 Preferably the sequence of peptide factor is modified
25 from the natural sequence to protect the peptides from
26 protease attack.

27
28 Preferred substitutions include the use of tyrosine
29 analogues at position 5 and arginine analogues at
30 position 9.

31
32 Preferably the peptide factor is capped at the N
33 terminal with an acetyl group.

34
35 Preferably the peptide factor is capped at the C
36 terminal with an amide group.

1 Preferably the thiol groups of cysteines are capped
2 with acetamido methyl groups.

3

4 In one embodiment the synthetic peptide has the
5 sequence

6

7 Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]NH₂

8

9 A preferred tyrosine analogue is Tic-OH.

10

11 A preferred arginine analogue is Citrulline.

12

13 The structure of Citrulline and other potential
14 arginine analogues are shown below.

15

16 Citrulline and analogues

17

18

19

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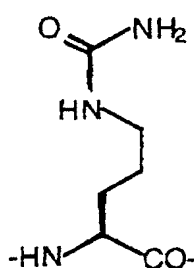
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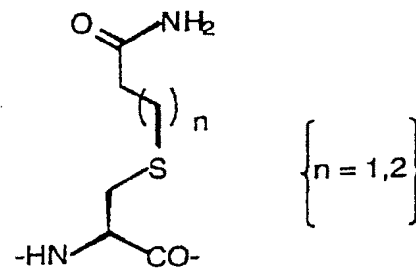
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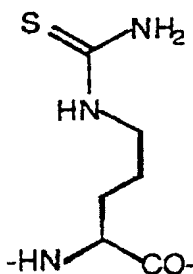


citrulline

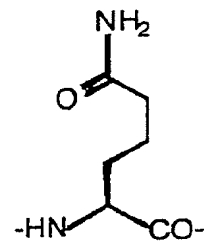


cysteine-derived analogues

{prepared by reaction of cysteine with
Br-(CH₂)_n-CONH₂}



thiono-citrulline



homo-glutamine

{prepared by reaction of ornithine with ammonium
isothiocyanate}

1 Preferably the peptide is truncated to a shorter
2 peptide without losing its antagonistic character.

3

4 The invention further provides a peptide agonist.

5

6 The agonist may be the native sequence (single letter
7 amino acid code:CDPGYIGSR-NH₂) or may have the tyrosine
8 substituted by any of a variety of tyrosine analogues
9 such as the conformationally restricted Tic-OH or
10 2',6'-dimethyl-beta-methyl-tyrosines, 2-O-methyl and 2-
11 O-ethyl-tyrosine and the like.

12

13 The agonist may be useful in healing endothelial cell
14 wounding.

15

16 For example, corneal endothelial cells can be damaged
17 during cataract operations and this damage does not
18 self-repair because these endothelial cells do not
19 divide. Healing can only be effected by cell migration
20 and spreading, and this may be promoted by the agonist.

21

22 In order to explore possible conformations for the
23 parent mEGF₃₃₋₄₂ peptide, it was modelled using molecular
24 dynamics. Based on these conformations a strategy has
25 been predicted to provide proteolytic protection by
26 being able to identify residues that are important to
27 the maintenance of a three-dimensional conformation
28 essential for 67LR recognition.

29

30 The following is a description of some examples of
31 modifications and uses of the invention.

32

33 1. On the basis of the modelled structures, it was
34 found that the arginine residue participated in H-
35 bonding, and speculated that this charge may not
36 be important. A peptide was synthesised based on

1 mEGF₃₃₋₄₂, in which the arginine residue at position
2 41 was replaced by citrulline (an uncharged
3 arginine mimetic with similar H-bonding
4 potential). This peptide provided to act as a
5 more potent 67LR antagonist and was found to be
6 resistant to trypsin degradation.

- 7
- 8 2. Double substitution of tyrosine₃₇ with Tic-OH and
9 arginine₄₁ with citrulline, to produce a mEGF₃₃₋₄₂-
10 derived peptide resistant to both chymotrypsin-
11 like and trypsin-like proteases.
- 12
- 13 3. Replacement of susceptible peptide bonds in mEGF₃₃₋₄₂
14 with protease-resistant peptide bond isosteres
15 (such as thionoamide or methylene amino bonds).
16
- 17 4. Conformationally restricted analogues may give
18 improved potency due to the essential 3-
19 dimensional conformation being stabilised. For
20 example, it should be possible to increase the
21 rigidity of the molecule by replacing each of the
22 central glycine residues in turn by α,α -dialkyl
23 substituted amino acids such as α -amino isobutyric
24 acid (AIB) or aminocyclopropane carboxylic acid
25 (ACPCA). Alternatively, the helical turn (which
26 we have identified as essential) could be
27 stabilised by bridging with suitable intra-chain
28 linkers, such as a disulphide bond between N- and
29 C-terminal [D] or [L]-cysteines.

30

31 **EXAMPLE 1**

32

33 The invention is demonstrated with reference to
34 the following figures wherein.

35

36 Figure 1a depicts a flat mount retina showing the

1 effects of ROP and Figure 1b depicts a retina from
2 laminin-agonist treated mouse showing re-
3 canalisation of vessels.

4

5

6 Treatment of Retinopathy of Prematurity (ROP)

7

8 Severely premature babies are at risk of
9 developing retinopathies due to their being
10 exposed to high oxygen levels post-partum. This
11 life-saving intervention compensates for poor lung
12 development but has the unfortunate side-effect of
13 causing unnaturally hyperoxic conditions in the
14 retina. The direct effect of this is to remove
15 the normal hypoxic cues for endothelial migration,
16 resulting in inhibition of capillary growth and
17 vaso-obliteration. When these babies are returned
18 to room air, hypoxic stimuli are restored and
19 retinal angiogenesis is again induced. However,
20 the newly induced angiogenesis is chaotic and
21 uncontrolled, often resulting in abnormal
22 penetration of vessels into the vitreous (see
23 Figure 1a, below). It is the uncontrolled growth
24 of these blood vessels that ultimately leads to
25 loss of visual activity.

26

27 It has now been shown that laminin agonist
28 treatment can reverse the effects of both hyper-
29 oxia induced vaso-ablation as well as norm-oxia-
30 induced angiogenesis in a murine model of
31 retinopathy of prematurity (ROP). In this model,
32 development of ROP can be prevented by treatment
33 of neonates with daily injections
34 (intraperitoneal) of 10 μ g of synthetic laminin β -
35 1₉₂₅₋₉₃₃ (also referred to as laminin B1₉₂₅₋₉₃₃, single
36 letter amino acid code:CDPGYIGSR-NH₂). See Figure

1 1b in comparison with 1a. Treatment with laminin
2 agonist (Figure 1b) prevents the uncontrolled
3 angiogenic response of ROP (Figure 1a) and
4 promotes re-canalisation of areas of vaso-
5 obliteration.

6

7 The invention is demonstrated with reference to
8 the following figures wherein Figure 1a depicts a
9 flat mount retina showing the effects of ROP

10

11 Figure 1b depicts a retina from laminin-agonist
12 treated mouse showing re-canalisation of vessels.

13

14 Murine model of proliferative retinopathy

15

16 Litters of 7 day old C57-BL/6J mice, together with
17 their nursing dams, are exposed to 80% oxygen in
18 an incubator maintained at 23°C and with a gas
19 exchange of 1.5L/min for 5 days according to the
20 protocol described by Stitt et al. (1998). On
21 postnatal day 12 (P12) the animals are returned to
22 room air and sacrificed at various times post-
23 hyperoxia. Animals are treated with daily i.p.
24 injections of either laminin agonists (10µg per
25 head per day) or vehicle control. Groups of room
26 air controls are maintained in parallel with
27 hyperoxia-exposed animals. Home Office project
28 and personal licenses are held for this work. All
29 animals are housed and maintained in accordance
30 with the ARVO regulations for animal care in
31 research.

32

33 Animals are sacrificed at pre-determined key
34 stages in the vaso-obliteration (P7-P12),
35 ischaemia (P12 onwards) and vaso-proliferative
36 responses (P12-21). At sacrifice, terminally

1 anaesthetised animals have a single eye enucleated
2 and the retina removed to be snap-frozen for later
3 RNA-extraction (see below). The fellow eye is
4 either perfused with fluorescein dextran or
5 enucleated and fixed in 4% paraformaldehyde for
6 histology, immunohistochemistry and *in situ*
7 hybridisation.

8

9 ALTERNATIVE USES

10

11 1. **Treatment of corneal wounds**

12

13 The cornea is a delicate transparent structure.
14 Being avascular, corneal wound healing depends
15 upon local self-renewal of the corneal epithelium.
16 This, in turn, depends upon the presence of a
17 mitogenically functional stem cell population
18 ('limbal cells'), which produce replacement cells
19 that migrate and desquamate at the denuded area.
20 Damage to these underlying stem cell populations
21 causes inappropriate re-epithelialization by
22 conjunctival cells followed by matrix deposition
23 and scar formation. The damaging agent may be
24 corrosive chemical or heat burns, erosion by
25 contact lenses, Stevens Johnson disease.

26

27 It is known that transplantation of limbal cell
28 autografts from the unaffected eye can restore a
29 stable healing of the corneal epithelium (Kenyon
30 et al., 1996). It has been proposed that
31 harvesting small samples of limbal stem cells,
32 followed by serial culture *in vitro* would provide
33 greater chance of success (particularly when both
34 eyes are affected) De Luca, et al., 1997).
35 However, with both protocols, correct uptake and
36 controlled migration of these grafted cells into

1 the corneal epithelium has not been optimised.

2

3 We propose that laminin agonists could be used to
4 stimulate the migratory response of the cells
5 prior to grafting, or alternatively topical
6 application of laminin agonists to the wound site
7 could be used to direct migration of the grafted
8 cells to the correct (denuded) area of the cornea.

9

10 2. Some microbial pathogens such as *Candida albicans*,
11 express 67LR and use this as a means of attaching
12 to human basement membranes. It is conceivable
13 that such infections could be abolished by
14 treatment with mEGF₃₃₋₄₂-derived peptides, which
15 would prevent the microbes from adhering to the
16 host.

17

18 **EXAMPLE 2**

19

20 **Peptide Study**

21

22 The purpose of the investigation was to determine the
23 molecular target of mEGF₍₃₃₋₄₂₎ and to identify the amino
24 acids that are essential for receptor recognition. In
25 addition, the key residues which confer laminin
26 antagonism on mEGF₍₃₃₋₄₂₎ were examined.

27

28 Two lead compounds were investigated; synthetic laminin
29 β -1 sequence CDPGYIGSR-NH₂ and mEGF₍₃₃₋₄₂₎ sequence
30 AcC(Acm)-VIGYSGDRC-(Acm)-NH₂. Bearing in mind the pure
31 antagonism of the murine EGF peptide, the aims of this
32 study were to identify the key residues responsible for
33 these contrasting activities using alanine scanning, in
34 the context of developing anti-angiogenic drugs for
35 retinopathy treatment.

36

1 In addition, using residue exchange between the two
2 peptides and molecular modelling to predict three-
3 dimensional structure, we wished to further investigate
4 the role of individual mEGF₍₃₃₋₄₂₎ residues in laminin
5 antagonism. A logical series of peptides was
6 synthesised and screened for receptor interaction, cell
7 adhesion and motility properties (Table 1a and 1b).

8

9 MATERIALS AND METHODS

10

11 Peptide synthesis

12

13 Peptide sequences based on and mEGF₍₃₃₋₄₂₎ were
14 synthesised on a model 432A peptide synthesizer
15 (Applied Biosystems, Warrington, UK), using standard
16 solid-phase Fmoc procedure (Fields 1990). Synthesis of
17 the peptides required successive additions of
18 derivatized amino acids to form a linear product.

19

20 Peptides were purified after synthesis using reverse
21 phase HPLC and purity confirmed by automated amino acid
22 analysis and electrospray mass spectrometry. All
23 peptide sequences were stored in the presence of
24 desiccant at -20°C until required for biological assay.

25

26 Laminin receptor antibody production

27

28 a. Preparation of MAPs

29

30 The peptide sequence (PTEDWSAQPATDWSAAPT),
31 corresponding to the COOH-terminal end of the human
32 laminin receptor, was used as the antigen template.
33 Derivation of the peptide, based on a CN-Br cleavage
34 fragment of the cDNA sequence encoding human laminin
35 receptor, has been described elsewhere (Wewer et al
36 1986). The antigen was synthesised as an octomeric

1 peptide derivative (MAPs) using automated Fmoc
 2 procedure (Tam 1988).

3

4

5 Table 1a: Peptide substitution

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
I	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Asp	Arg	ACM Cys-NH ₂
II	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Ser	Arg	ACM Cys-NH ₂
III	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Ser	Arg	ACM Cys-NH ₂
IV	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Cit	ACM Cys-NH ₂
V	acetyl	ACM Cys	Val	Ile	Gly	OH Tic	Ser	Gly	Asp	Arg	ACM Cys-NH ₂

Table 1b: Peptide substitution (alanine scanning)

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VI	acetyl	ACM Cys	Val	Ala	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VII	acetyl	ACM Cys	Ala	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VIII	acetyl	Ala	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
IX	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	Ala-NH ₂
X	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ala	Gly	Asp	Arg	ACM Cys-NH ₂

1 **b. Immunisation schedule**

2
3 A pre-immune test bleed (5ml) was obtained from the
4 marginal ear vein of a male New Zealand White rabbit
5 (3.2 kg). The bleed was allowed to clot for 2 h at room
6 temperature after which its edge was detached from the
7 wall of the collection vessel. The clot was then
8 allowed to contract overnight at 4°C. Serum was then
9 removed and the residual material pelleted out by
10 centrifugation (10 min at 2,500 g). Extracted serum
11 (3.5 ml) was then frozen at -20°C until required.

12
13 Immunogen was prepared by the emulsion of MAPs (0.5 g
14 antigen in 0.5 ml PBS) in an equivalent volume of
15 adjuvant (Alum Imject; Pierce, Chester, UK). The
16 animals immune system was primed by introducing
17 immunogen (50 µg) through subcutaneous injection at
18 different sites on the animals back. The rabbit was
19 boosted by both subcutaneous and intramuscular
20 injection, 21 days after priming, using an increased
21 dose of immunogen (800 µg). Subsequent boosts were
22 performed by intramuscular injection after a further 14
23 days (800 µg immunogen), and thereafter at 21 day
24 intervals. Test bleeds were taken 2 days after each
25 boost and the serum extracted as described above. The
26 animal was boosted and bled a total of three times.

27
28 **c. Enzyme-linked immunoabsorbent assay**

29
30 ELISA was used to determine the specificity of the
31 antibody prepared against the synthetic MAPs peptide
32 and to determine the efficacy of binding with respect
33 to that of the linear precursor.

34
35 Peptides were dissolved in distilled water and diluted

1 to 10 $\mu\text{g/ml}$ in coating buffer. Aliquots (100 μl) of
2 either linear or MAPs peptide were then added to the
3 wells of microtitre plates (Microtest III; Becton
4 Dickinson Ltd., Oxford, UK) and incubated overnight at
5 37°C. The wells were then rinsed with 100 μl wash
6 buffer and air dried. Excess adsorption sites were
7 blocked (1 h incubation at 22°C) by the addition of 10%
8 casein in PBS (0.1 ml/well). Subsequent to the removal
9 of casein solution by aspiration, wells were again
10 rinsed with wash buffer and air dried.

11

12 Antisera or pre-immune sera were then serially diluted
13 in PBS and 100 μl of each incubated in peptide coated
14 wells for 1 h at 37°C. After rinsing (0.1 ml wash
15 buffer), 100 μl per well of 5 $\mu\text{g/ml}$ secondary antibody
16 (horse-radish peroxidase-conjugated goat anti-rabbit
17 IgG; Amersham International, Aylesbury, UK) was added
18 to each well and the plates incubated at 37°C for 1 h.

19

20 Wells were again rinsed with wash buffer and 0.1 ml
21 substrate solution (TMB peroxidase) added to each. The
22 plate was then incubated at 22°C for 30 min and the
23 colour reaction stopped by the addition of 0.5M H_2SO_4
24 (0.1 ml/well). Absorbance was measured at 450 nm on a
25 Titertek Multiscan plate reader.

26

27 d. Purification of IgG fraction

28

29 Anti-laminin receptor antiserum was purified using
30 immobilised protein G-sepharose columns (Pharmacia
31 Biotech, Uppasla, Sweden). The columns were
32 equilibrated with 20 ml sodium phosphate buffer (pH
33 7.0). Antiserum was diluted 1:4 in the same buffer and
34 a 1 ml aliquot loaded onto the column (flow rate 150
35 ml/h, fraction size 2.5 ml). After exclusion of the

1 unbound fraction, as determined by absorbance at 280nm,
2 the IgG component of the antiserum was eluted with 0.1M
3 glycine-HCl (pH 2.7), into tubes containing 0.1 ml Tris
4 (1M), pH 9.0. The eluted IgG fractions were bulked and
5 stored at -20°C until required.

6

7 **Maintenance of cell cultures**

8

9 Cancer and endothelial cells were maintained in either
10 DMEM (T47-D) or RPMI (SK HEP-1) media, supplemented
11 with 10% FCS, 100 IU/ml penicillin and 100 µg/ml
12 streptomycin. Cells were incubated at 37°C in a
13 humidified atmosphere of 95% air: 5% CO₂ and media
14 refreshed as required. Cultures (at 80-85% confluence)
15 were routinely passed on removal from monolayer by the
16 action of trypsin (0.25%) and EDTA (0.02%) in CFS.

17

18 The viability of cell populations following
19 trypsinisation was determined by the trypan blue vital
20 dye exclusion test. Populations confirmed as being in
21 excess of 95% viable were used in all studies.

22

23 Media were screened for possible bacterial or fungal
24 contamination by incubating 1ml aliquots with both
25 nutrient and Saboraud dextrose broths (Oxoid Ltd.,
26 Basingstoke, UK). Cell populations were routinely
27 monitored for sub-clinical infections by periodically
28 culturing in the absence of antibiotics.

29

30 Both cell lines and media were examined for the
31 presence of contaminating *Mycoplasma* spp. by the method
32 of Chen (1977).

33 **Determination of cell numbers**

34

35 Single cell suspensions were quantified using an

1 automated counter (Coulter Electronics, Harpenden, UK).
2 A 1 ml aliquot of cell suspension was diluted 1 in 20
3 in Isoton and 0.5 ml samples counted. The mean of 5
4 counts was taken and the total number of cells
5 determined. Estimates of cell number were confirmed by
6 counting in a haemocytometer.

7
8 For microtitre end-point assays, cell numbers were
9 estimated from the crystal violet staining index of the
10 cell line (Kanamaru and Yoshida 1989). Briefly, after
11 removal of media from the assay system cells were fixed
12 with formaldehyde (10% in PBS), and washed with
13 distilled H₂O. Aliquots (100 μ l) of crystal violet
14 solution (0.1% in distilled H₂O) were added to each well
15 and the plates allowed to stand for 30 min. Excess
16 stain was removed by rinsing with distilled H₂O (3 x 100
17 μ l). The wells were then air-dried and the remaining
18 crystal violet extracted with 100 μ l acidified
19 methanol. Absorbance at 620 nm was determined using a
20 Titertek Multiscan spectrophotometer.

21

22 Proliferation assays

23

24 The effects of synthetic peptides and growth factors on
25 the growth of breast cancer and endothelial cells were
26 determined as detailed.

27

28 Exponentially growing cells were harvested by
29 trypsinisation, as previously described. After rinsing
30 and resuspending in the relevant culture media
31 (containing 10% FCS), the cells (100 μ l aliquots) were
32 dispensed into 96-well microtitre plates at a
33 population density of 2×10^4 cells/well (6 wells per
34 experimental condition). Cells were incubated for
35 24 h at 37°C after which the media was removed and the

1 wells rinsed with CFS (3 x 100 μ l), to rid the plates
2 of cells in suspension. Media was then replaced with
3 that containing the relevant controls or treatment
4 supplements as detailed in individual experiments.

5
6 Cell numbers were evaluated spectrophotometrically at
7 620 nm, over the period of assay, after fixing with 10%
8 formaldehyde and staining with crystal violet.

9
10 Proliferative responses were analysed using the
11 Wilcoxon Rank test and significant differences at the p
12 < 0.05 level, defined. Results of all growth studies
13 were confirmed in at least 3 individual experiments.

14
15 **Laminin attachment assay**

16
17 Non-tissue culture grade 96-well plates, coated with
18 2.5 μ g murine laminin in 50 μ l CFS per well, were air-
19 dried overnight at room temperature. Preliminary
20 experiments indicated that cell attachment was
21 concentration dependent; maximal binding occurred at a
22 laminin coating of 2.5 μ g/well. After rinsing with CFS
23 (100 μ l), the plastic was saturated with casein (0.2%
24 in CFS). Plates were incubated at room temperature for
25 45 min then washed extensively with CFS (3 x 100 μ l).

26
27 After removal of culture media, cells were detached
28 from monolayers by the action of EGTA (0.02% in CFS) at
29 37°C. The cells were then centrifuged at 800 g for 2
30 min and the pellet resuspended in DMEM (T-47D) or RPMI
31 (SK HEP-1).

32
33 Cells, at a population density of 10^6 cells/ml, were
34 then aliquoted (1 ml) into microfuge tubes containing
35 the individual peptide sequences and incubated for 1 h

1 at 37°C. The cells (100 µl aliquots) were then added to
2 the pre-coated multi-well plates and incubated for a
3 further 60 min. Incubation media were removed and the
4 wells washed with CFS (3 x 100 µl) to rid the plates of
5 non-adherent cells.

6
7 Attached cell numbers were evaluated
8 spectrophotometrically at 620 nm after fixing with 10%
9 formaldehyde and staining with crystal violet.

10

11 **Attachment to mEGF₍₃₃₋₄₂₎**

12

13 That mEGF₍₃₃₋₄₂₎ bound to the 67kDa laminin receptor was
14 demonstrated using a biotinylated derivative of the
15 peptide (Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]-K-[N^ε-
16 biotin]-amide) and a modification of the above laminin
17 attachment assay.

18

19 Briefly, 96-well plates were coated with 100 µl/well
20 streptavidin (5 µg/ml in carbonate buffer pH 9.6) and
21 following an overnight incubation at 37°C, wells were
22 washed with CFS (3 x 100 µl) and the plastic blocked
23 with casein (0.2% in CFS). The plates were then
24 incubated at room temperature for 45 min and washed
25 with CFS as previously detailed. Biotinylated mEGF₍₃₃₋₄₂₎
26 in CFS was then aliquoted into the wells (0.1 ml of 100
27 µM) and the plates incubated for 3 h at 37°C.

28

29 After a further block with 0.2% casein, the wells were
30 washed with CFS (3 x 100 µl aliquots). Plates were kept
31 at 4°C and used within 2 h.

32

33 Cells were prepared as above and pre-incubated for 1 h
34 at 37°C with serial dilutions of anti-laminin receptor
35 polyclonal (see below) or anti-EGF (R1) receptor

1 monoclonal antibodies. Subsequent procedures were as
2 detailed for the laminin attachment assay.

3

4 **Laminin receptor binding determinations**

5

6 **a. Radiolabelling of laminin**

7

8 ^{125}I -laminin was prepared using ^{125}I -labelled sodium
9 iodide (Amersham, UK) and immobilised chloramine-T
10 (Iodobeads; Pierce, Illinois). Prior to use, the beads
11 were washed with 500 μl phosphate buffer (pH 6.5) to
12 remove excess reagent from the support. These were then
13 allowed to air dry and individual beads added to a
14 solution of carrier free Na^{125}I , diluted with iodination
15 buffer (phosphate buffer pH 7.4). The beads were
16 allowed to equilibrate for 5 min.

17

18 Laminin (10 μg in 10 μl) was then diluted into the
19 iodination buffer and the system incubated at 20°C for
20 15 min. The solution was then removed from the reaction
21 vessel and excess Na^{125}I and unincorporated $^{125}\text{I}_2$
22 separated from the iodinated protein by gel filtration
23 on a GF-5 exclusion column (Pierce, Illinois).
24 Iodinated laminin fractions were recovered at a
25 specific activity of approximately 1.2 mCi/mg protein
26 (864 Ci/mmol).

27

28 **b. Competition binding estimation**

29

30 Near confluent cultures of T47-D or SK HEP-1 cells were
31 removed from monolayer with 0.02% EGTA and passed
32 through a G-25 syringe needle to produce single cell
33 suspensions. Aliquots of each cell type (10^6 cells/ml)
34 were dispensed into separate Ependorf tubes (1 ml each)
35 and pelleted. The cells were then resuspended in 1 ml

1 ice-cold RPMI (SK HEP-1) or DMEM (T47-D) containing
2 0.1% BSA and either laminin or synthetic peptide at the
3 concentrations indicated. Iodinated laminin was then
4 added to each cell suspension to give a final ^{125}I -
5 laminin concentration of 0.1 nM (approximately 50,000
6 cpm). These mixtures were incubated overnight at 4°C.

7
8 The tubes were then microfuged at 10,000 g and the
9 supernatant removed. After washing the pellet with 500
10 μl CFS, the remaining radioactivity was determined
11 using a gamma radiation counter. Non-specific binding
12 was determined by incubating cells with a 1000-fold
13 molar excess of unlabelled laminin. All estimations
14 were carried out in triplicate.

15
16 IC_{50} (concentration of unlabelled peptide required to
17 produce 50% inhibition of radioligand binding) and EC_{50}
18 (effective concentration for 50% inhibition of cell
19 attachment) values were calculated using the Grafit
20 curve-fitting programme (Erithacus Software, London,
21 UK).

22 23 **Migration assays**

24
25 The method used was basically as described by Albrecht-
26 Buehler (1977). Briefly, coverslips (22 x 22 mm) were
27 treated in 5% detergent (7X; ICN Biomedicals) and
28 washed in alcohol to remove grease. After drying, they
29 were immersed in gelatin solution (Sigma, 300 Bloom;
30 0.5 g in 300 ml distilled H_2O) for 10 min. The
31 coverslips were then dried by placing in a 70°C oven
32 for 45 min.

33
34 Colloidal gold suspension was prepared by adding 11 ml
35 distilled H_2O and 6 ml Na_2CO_3 (36.5 mM) to 1.8 ml AuHCl_4

1 (14.5 mM). The mixture was heated to 95°C at which
2 point 1.8 ml of freshly prepared 0.1% formaldehyde
3 solution was added; the temperature was maintained at
4 95°C. A suspension of colloidal gold was formed which
5 was brown to absorbed light and blue to transmitted
6 light.

7
8 The gold suspension, was then added to petri dishes
9 containing individual coverslips and the plates
10 incubated at 37°C for 45 min. After washing with CFS (3
11 x 4 ml) to remove unattached gold particles, the
12 coverslips were transferred to 6-well cluster dishes
13 and UV sterilised.

14
15 Endothelial cells (SK HEP-1 and BRCE) in culture media
16 (0.3 ml) were seeded onto the coverslips at an
17 approximate density of 5×10^3 cells per well. The cells
18 were allowed to plate down for 2 h at 37°C after which
19 the treatments were added. Assay systems were
20 maintained for a further 18 h after which the cells
21 were fixed using 3% gluteraldehyde in cacodylate buffer
22 (pH 7.2).

23
24 The assays were examined using a Leica DM1RB phase
25 contrast microscope and Q500MC image analysis system
26 incorporating a JVC TK-1280E colour camera (Leica,
27 Milton Keynes, UK). The track images of at least 30
28 cells were video-captured and the area (representing
29 migration response) determined for each. Statistical
30 analysis of these areas was then carried out using
31 Macintosh Instat software to perform both Kruskal-
32 Wallis analysis of variance and Mann-Whitney *U*-tests in
33 order to compare the treatment groups with controls.

34 35 RESULTS

1 **Proliferative response**

2

3 All peptides were examined for their ability to
4 influence the growth of T47-D and SK-Hep 1 cell lines.
5 At concentrations of peptide up to 100 μ M, no
6 significant effects were observed in either cell line.

7

8 **Mechanism of action**

9

10 It had shown previously that mEGF₍₃₃₋₄₂₎ could inhibit the
11 EGF-stimulated angiogenic response in the early chick
12 as well as blocking the basal and EGF-stimulated
13 motility of primary and established endothelial cells.

14

15 During the present study it is shown that mEGF₍₃₃₋₄₂₎ also
16 inhibits the angiogenic effects of laminin (Nelson et
17 al 1995). Furthermore, it is demonstrated that the
18 anti-angiogenic effects of mEGF₍₃₃₋₄₂₎ are mediated solely
19 through the high affinity 67 kDa laminin receptor (67-
20 LR) and not through the EGF receptor.

21

22 The study also confirms that mEGF₍₃₃₋₄₂₎, Lam. β -1₍₉₂₅₋₉₃₃₎ and
23 laminin are equipotent in ¹²⁵I-laminin displacement
24 receptor assays, and that both of the small peptidal
25 ligands have similar potencies in specific laminin cell
26 attachment assays.

27

28 In addition, it is shown that the commonly used chick
29 angiogenesis models are not appropriate to the study of
30 laminin mediated human angiogenesis: although it is
31 confirmed that Lam. β -1₍₉₂₅₋₉₃₃₎ acts as a partial laminin
32 antagonist in chick, it was found to be a pure agonist
33 in mammalian cell lines. This is a highly significant
34 point given that pharmaceutical companies (such as
35 Angiotech, Vancouver, BC) are using the chick CAM assay

1 as the sole screening method for the discovery of anti-
2 angiogenic lead compounds. This may be inappropriate
3 for use in human disease.

4
5 This study is the first to show that the YIGSR-receptor
6 is, in fact, the 67 kda high affinity laminin receptor
7 (67-LR). In collaboration with Professor Archer's team
8 at the Department of Ophthalmology, Royal Victoria
9 Hospital, Belfast, it has been determined that the 67-
10 LR is preferentially expressed in new vessels during
11 oxygen-induced retinopathy in neonatal mice.

12

13 Peptide antagonist development

14

15 The N-terminus of Lam. β -1₍₉₂₅₋₉₃₃₎ is not necessary for
16 receptor recognition and the agonist activity of YIGSR
17 peptide (Ostheimer et al 1992, Kawasaki et al 1994).

18

19 However, alanine scanning of the starting peptide
20 (mEGF₍₃₃₋₄₂₎) indicated that residues at positions 1, 2,
21 3, and 6 (peptides VI, VII, VIII and X respectively),
22 are essential for receptor mediated activities as
23 determined by ¹²⁵I-laminin displacement and cell
24 attachment to laminin through the 67-LR. Substitution
25 of these individual residues by alanine leads to a
26 dramatic decrease in receptor affinity observed as an
27 increased IC₅₀ (Table 2) and a parallel decrease in
28 their ability to block adhesion to laminin (increased
29 EC₅₀; Table 2). Characterisation of these analogues with
30 regard to effects on motility, largely confirmed these
31 findings although there was one exception; peptide
32 VIII. Results from the migration assay identified this
33 sequence (alanine for cysteine (P1)) as being a weak
34 laminin agonist despite there being a much reduced
35 response in the other two assays. It is suggested that

1 this peptide may influence laminin receptor mediated
2 migration through an alternative mechanism (Scott
3 1997) .

4
5 Substitution at P10 (alanine for cysteine (peptide X)
6 retains both receptor binding and adhesion displacing
7 activities but has the effect of changing the
8 antagonistic parent into an agonist analogue. This
9 reflects the response the agonism of Lam. β -1₍₉₂₅₋₉₃₃₎ ,
10 which also lacks the C-terminal cysteine, and suggests
11 that this cysteine is not essential for receptor
12 recognition, but is required for antagonism of mEGF₍₃₃₋
13 42) .

14
15 Studies have reported that the positive charge offered
16 by arginine (P9) is essential for the biological
17 activity of Lam. β -1₍₉₂₅₋₉₃₃₎ (McKelvey et al 1991, Kawasaki
18 et al 1994). Glutamate substitution for arginine
19 generates a negative charge at this position with
20 corresponding loss of biological activities (Kawasaki
21 et al 1994) .

22
23 However, the substitution of arginine (P9) with
24 positively-charged lysine (McKelvey et al 1991) also
25 results in complete loss of ligand binding and
26 biological activities, suggesting that the mere
27 presence of a positive charge at this position is, in
28 itself, insufficient for receptor recognition. This
29 modelling studies suggest that H-bonding of the
30 guanidino group of the arginyl residue to the aromatic
31 sidechain of the tyrosyl residue (P5) in the consensus
32 sequence GYXGXR presents an acceptable motif for 67-LR
33 activation by both mEGF₍₃₃₋₄₂₎ and Lam. β -1₍₉₂₅₋₉₃₃₎ .

34
35 Substitution of tyrosine (P5) with a conformationally

1 restricted mimetic (tetrahydroisoquinoline-3-carboxylic
2 acid; Tic-OH) in peptide V converted the antagonist
3 mEGF₍₃₃₋₄₂₎ into an agonist. This residue substitution
4 generates a predicted conformation unlikely to be able
5 to form H-bonds. Although both receptor binding and
6 adhesion responses were retained in this peptide the
7 loss of antagonism would suggest that H-bonding between
8 tyrosine (P5) and the arginine (P9) is important for
9 these antagonist activities.

10

11 Modelling studies suggested that citrulline (an
12 uncharged arginine mimetic) would also be capable of
13 forming this H-bonded motif.

14

15 It was found that replacement of arginine (P9) with
16 citrulline (peptide IV) increased both receptor binding
17 and inhibition of attachment to laminin substrata
18 whilst retaining antagonist migratory response (Table
19 2), reinforcing the observation that it is not the
20 positive charge that is required rather than an active
21 conformation generated by hydrogen bonding. These
22 findings thus identify H-bonding between P5 and P9 as
23 being more important than the charge at the P9 arginine
24 in determining antagonist activity.

25

26 Subsequent strategies involved the substitution of
27 variant residues in the antagonistic mEGF₍₃₃₋₄₂₎ with
28 those present in the agonistic Lam.β-1₍₉₂₅₋₉₃₃₎ sequence
29 (peptides I-III), in an effort to identify key amino
30 acids in the C-terminal regions (P5-10) of the two
31 ligands responsible for their contrasting
32 bioactivities.

33

34 Substitution of isoleucine (P6) for serine (peptide I)
35 resulted in both reduced receptor affinity and potency

1 in displacement of cell adhesion to laminin. However,
2 this analogue retained weak antagonist activities in
3 the motility assay. It is therefore of interest that
4 studies on the YIGSR sequence indicate that residue
5 substitution, at the position taken by isoleucine in
6 the pentapeptide, are well tolerated and may increase
7 potency (Kawasaki et al 1994).

8
9 Replacement of aspartate (P8) with serine (peptide II)
10 resulted in a complete loss of biological function. as
11 did peptide III encompassing both the former
12 (isoleucine (P6) for serine) and latter (serine (P8)
13 for aspartate) substitutions. Since this mEGF₍₃₃₋₄₂₎
14 analogue sequence (peptide II) encompasses the active
15 YIGSR amino acid sequence agonist, it is suggested that
16 this loss of activity may be attributed to the valine
17 (P2) and isoleucine (P3) residues in the N-terminal
18 half of mEGF₍₃₃₋₄₂₎. Alternatively, addition of a C-
19 terminal cysteine to the YIGSR sequence is known to
20 reduce potency (Kawasaki et al 1994). Additional
21 peptides incorporating the valine (P2) and isoleucine
22 (P3) substitutions are currently under investigation.

23
24 The determination of the minimum core peptide structure
25 is ongoing and involves similar characterisation
26 studies on a number of sequences truncated at the C-
27 terminal.

28
29 These studies have thus identified an important
30 antagonist of 67-LR mediated activities in peptide IV.
31 The sequence, (AcC(Acm)-VIGYSGD-[Cit]-C-(Acm)-NH₂.), may
32 provide an important template for anti-angiogenic drugs
33 in that it is resistant to cleavage by trypsin-like
34 proteases and has been identified as being more potent
35 than mEGF₍₃₃₋₄₂₎ in screening procedures.

1 Advantages

2

3 The advantages of the invention, and the ways in which
4 disadvantages of previously known arrangements are
5 overcome include:

6

7 1. Unlike the native 67LR ligand (laminin β -1₉₂₅₋₉₃₃),
8 which is angiogenic in human models, the mEGF₃₃₋₄₂-
9 derived agents are anti-angiogenic in human
10 models.

11

12 2. mEGF₃₃₋₄₂ has the advantage of inhibiting both
13 laminin- and EGF-stimulated angiogenesis.

14

15 3. mEGF₃₃₋₄₂ prevents tumour cell attachment to
16 basement membranes.

17

18

1 CLAIMS

2

3 1 Use of a peptide factor derived from amino acid
4 residues 33 to 42 of murine epidermal growth or a
5 synthetic equivalent thereof in the preparation of a
6 medicament to target laminin receptors.

7

8 2 A peptide factor derived from amino acid residues 33
9 to 42 of murine epidermal growth factor peptide or a
10 synthetic equivalent thereof wherein the peptide
11 factor is modified to protect it from proteolytic
12 degradation and the peptide binds to laminin
13 receptors.

14

15 3 A peptide factor as claimed in claim 2 wherein the
16 modifications consist of at least one modification
17 chosen from the group comprising; substitution of
18 tyrosine by tyrosine analogues, substitution of
19 arginine by arginine analogues, capping the N
20 terminal of the peptide capping the C terminal of the
21 peptide and capping thiol groups of cysteines.

22

23 4 A peptide as claimed in claim 2 or claim 3 wherein
24 the peptide has the sequence.

25

26 Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]NH₂

27

28 5 A peptide as claimed in claim 3 wherein tyrosine is
29 substituted by Tic-OH.

30

31 6 A peptide as claimed in claim 3 wherein arginine is
32 substituted by Citrulline.

33

34 7 Use of a peptide factor as claimed in claim 1 in the
35 preparation of a medicament to bind laminin receptors

- 1 as an antagonist.
2
3 8 Use of a peptide factor as claimed in claim 1 in the
4 preparation of a medicament to bind laminin receptors
5 as an agonist.
6
7 9 Use as claimed in claim 8 in the preparation of a
8 medicament for healing endothelial cell wounding.
9
10 10 Use as claimed in claim 8 or 9 for the treatment of
11 retinopathy of immaturity.
12

Fig 1a

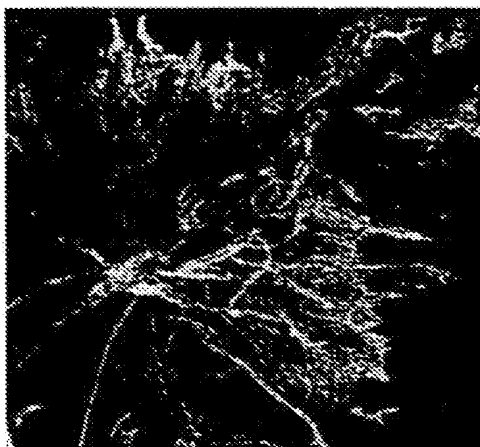
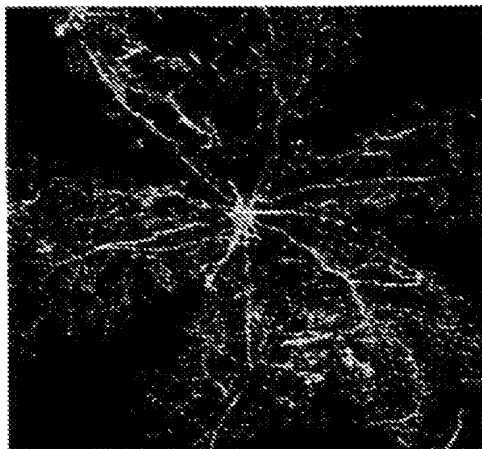


Fig 1b



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01211

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/485 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 686 283 A (NESTOR JR JOHN J ET AL) 11 August 1987 (1987-08-11) claims; examples ---	1-3,7-10
X	BAILIE, J. R. ET AL: "Synthesis and receptor-binding activity of peptide fragments of epidermal growth factor" BIOCHEM. SOC. TRANS. (1989), 17(2), 409-10 ,1989, XP002116635 page 409, right-hand column; table 1 ---	1-4,7-10
X	MCFERRAN, NEIL V. ET AL: "A peptide molecular dynamics study correlates structure with function" BIOCHEM. SOC. TRANS. (1996), 24(1), 127S , 1996, XP002116636 page 127S, left-hand column, paragraph 4 --- -/--	1-4,7-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NELSON, JOHN ET AL: "Murine epidermal growth factor (EGF) fragment (33-42) inhibits both EGF- and laminin-dependent endothelial cell motility and angiogenesis" CANCER RES. (1995), 55(17), 3772-6 ,1995, XP002116637 page 228 -----	1-3,7-10
X	NELSON, JOHN ET AL: "Murine epidermal growth factor peptide (33-42) binds to a YIGSR-specific laminin receptor on both tumor and endothelial cells" J. BIOL. CHEM. (1996), 271(42), 26179-26186 ,1996, XP002116638 page 26183, left-hand column, paragraph 3 -page 26186, left-hand column, last paragraph -----	1

Information on patent family members

PCT/GB 99/01211

Form PCT/ISA/210 (patent family annex) (July 1992)